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ANTIBODIES IN AIR ASSAY: APPLICATION OF ELISA TO AEROSOL RESEARCH

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December 1996

Aberdeen Proving Ground, MD 21010-5423

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This report addresses the analytical methodology used in the Antibody in Air Assay Study. Tests were performed to determine the stability of antibodies, retention of antibody active binding sites, and antibody binding in an aerosol. Quantitation of antibody and determination of active binding sites were assessed by enzyme-linked immunosorbent assay (ELISA).

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EXECUTIVE SUMMARY

The Antibody in Air Assay study was initiated to determine whether antibodies could be successfully aerosolized and retain their binding capacity. This report addresses the analytical methodology used in the study. Tests were performed to determine the stability of antibodies, retention of antibody active binding sites, and antibody binding in an aerosol. Quantitation of antibody and determination of active binding sites were assessed by enzyme-linked immunosorbent assay (ELISA).

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PREFACE

The work described in this report was authorized under Project No. 1O162622A553, CB Defense and General Investigation. This work was started in September 1993 and completed in September 1995.

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CONTENTS

FIGURES

TABLES

ANTIBODIES IN AIR ASSAY: APPLICATION OF ELISA TO AEROSOL RESEARCH

1. INTRODUCTION

Antibodies are large globular serum proteins (150 kD) with the ability to bind to a specific antigen. They are produced by B-lymphocytes in mammalian immune systems upon introduction and recognition of foreign material (antigen) in the body. Each antibody that is formed has a unique three dimensional structure that recognizes and binds to epitopes on that particular antigen. This specificity of interaction with the antigen is well known from the use of vaccines for active immunity disease prevention, and has led to the *in vivo* use of antibodies for passive immunity anti-sera[1,2], in low velocity aerosols for immunoprophylaxis of infectious disease[3,4] and immunotherapy in respiratory tract diseases[5,6]. In addition, antibody-antigen interactions occur *in vitro* for solid phase detection using Enzyme-Linked Immunosorbent Assay (ELISA)[7,8] and biosensors[9-12]. In these *in vitro* systems, antibodies retain a remarkable degree of stability and sensitivity even though they are removed from their native environment and attached to a solid surface. A literature search on use of ELISA in testing aerosol samples revealed that it has been used for identification of bacteria and pollen[13-15], and determination of the stability of aerosolized ovalbumin[16]. This paper deals with the use of ELISA as a probe of antibody interactions in the Antibody in Air Assay.

The ELISA was chosen as a test assay because it is sensitive (ng/ml) and well established for detection of either an antigen or antibody target molecule. In the assay, the target molecule is immobilized in the microwells, washed to remove excess and incubated with an anti-target antibody conjugated to an enzyme. In the presence of specific compounds, the enzyme produces a colorimetric change that is directly correlated to the amount of target present. The purpose of this study was to identify the following effects of aerosolization of antibodies: 1) antibody integrity-do antibodies retain enough structural integrity to be recognized by a secondary antibody, 2) antibody binding- do antibodies retain binding specificity (intact binding sites) for an antigen and 3) antibody/antigen binding in an aerosol- do sufficient aerosol droplets containing either antibody or antigen coalesce during circulation in the chamber to produce a measurable antibody/antigen binding that occurs in droplets in the aerosol phase. The aerosol aspects of this study will be discussed in a separate report.

2. MATERIALS AND METHODS

2.1 Chemicals Used.

Rabbit IgG antibody (RAb) was obtained from Pentex, Inc. (Kankakee, IL), peroxidase-labeled anti-rabbit (POD-anti-RAb) and

2,2'-Azino-bis (3-Ethylbenzthiazoline-6-sulfonic acid) (ABTS) peroxidase substrate were obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Anti-RAb and bovine serum albumin (BSA) were obtained from Sigma Chemical Company (Saint Louis, MO) and anti-BSA was from Calbiochem (San Diego, CA). Anti-RAb-labeled BioMag® paramagnetic beads (magbeads) were obtained from PerSpective Diagnostics (Cambridge, MA). All other chemicals were of analytical grade and were obtained from Sigma Chemical Company and Calbiochem.

2.2 Preparation of Bioreagents.

Test solutions, RAb (2 mg/ml stock solution) and anti-BSA were dissolved in phosphate buffered saline (PBS, pH 7.4) containing 15 μg/ml sodium fluorescein. Peroxidase label was prepared by diluting POD-anti-RAb in PBS containing 1% skim milk. Magbeads (1 mg/ml) were washed and resuspended in PBS prior to aerosolization.

2.3 Sample Aerosolization and Collection.

Test solutions for the first two tests were prepared as previously described[17], briefly, antibody solution was aerosolized using a compressed air atomizer and the resulting aerosol collected directly into 20 ml chilled PBS in an all glass impinger (AGI-30).

For aerosol binding studies, a method was required which would allow collection of droplets that had coalesced during the aerosol phase. AGI collection was ruled out since it would be impossible to determine if antibody/antigen binding occurred within aerosol droplets or in solution after the droplets were collected. It was therefore determined that a magnetic capture method was required. This technique is widely used for capture of magbead/Ab complex from aqueous media by placing the container on a magnet, drawing the magbead/Ab complex to one side and decanting the remaining liquid. In an aerosol, separation could be accomplished by drawing the aerosol across a tape-covered magnet. Droplets containing magbead and magbead/Ab complex would be captured on the tape while droplets containing only Ab would pass. A Magnetic Particle Separator (MPS) and collection method has been described[18]. Briefly, Magbeads and RAb were simultaneously sprayed into the chamber, passed over a neutralizer to remove any static charge, and collected in MPS. Magnets were lined up to correspond to the holes in a microwell plate and were overlayed with clear plastic tape to which magnetic particles adhered. The collecting tape was removed and affixed to the bottom of the microwell plate in which well bottoms were drilled out, i.e., the tape was used as the well bottom (Figure 1).

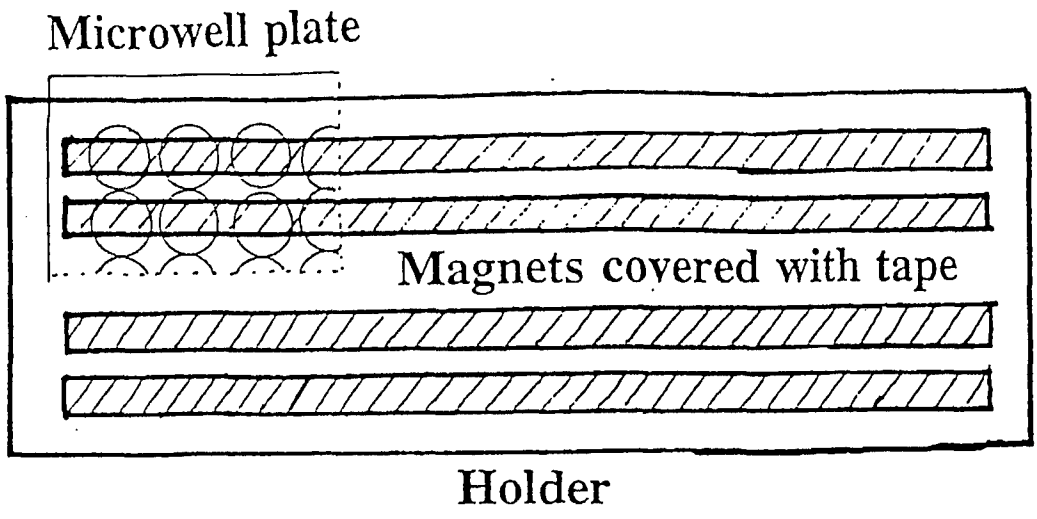

Figure 1. Diagram of magnetic particle collector with microwell plate superimposed.

2.4 Antibody Quantitation.

Sample antibody activity was determined by enzyme-linked immunosorbent assay (ELISA) using linear regression analysis of known concentrations of RAb. Briefly, Ab or Ab complexes were adsorbed to 96-well microwell plates for 1 hr at room temperature, washed twice with PBS containing blocker, and incubated with POD-anti-RAb for 1 hr. After washing the cells, 100 μl ABTS substrate was pipetted into all wells and optical density OD_{405} read on a Dynatech MR5000 spectrophotometer after 10 min. Abs were quantitated by comparison with known concentrations using serial dilution techniques and linear regression analysis. Figure 2 is a representative ELISA showing the color change with increasing amounts of RAb.

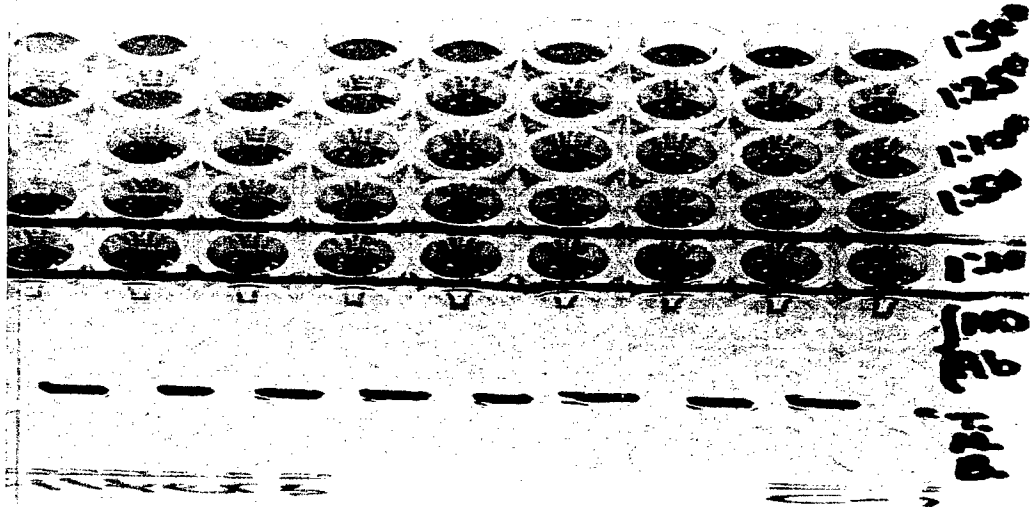

Figure 2. Representative ELISA.

2.5 Experimental Protocols.

2.5.1 Antibody integrity.

Several tests were run to determine the feasibility of binding of antibodies in an aerosol. The first test was the determination of the viability of an aerosolized antibody. Collected RAb was quantitated by analysis of ELISA data. A direct ELISA assay was used in which POD-anti-RAb was incubated with the RAb immobilized in the wells (Fig. 3).

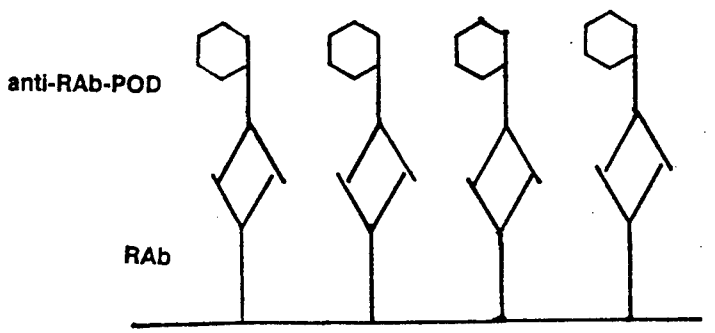

Figure 3. Schematic representation of direct ELISA assay.

2.5.2 Antibody Binding.

To determine whether Abs retain active sites after aerosolization, anti-BSA Ab was prepared as above. An ELISA capture assay was used in which microwell plates coated with antigen (0.01% BSA). The anti-BSA Ab bound to the BSA only if the active sites were intact. The added label was used for quantitation (Fig. 4).

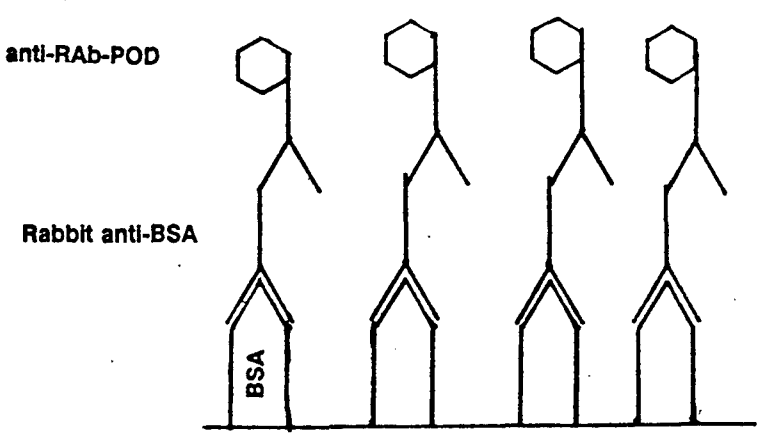

Figure 4. Schematic representation of capture ELISA assay.

2.5.3 Antibody/Antigen Binding in Aerosol.

Binding of an antibody in air was accomplished by collision of droplets containing RAb with droplets containing magbeads, thus forming magbead/Ab complex in coalesced droplets. Magbeads and RAb were atomized simultaneously and collected as described, and the amount of RAb present as well as nonspecific binding was quantitated by using a capture ELISA format. Half of the wells were preincubated with unlabeled anti-RAb to determine any nonspecific binding of the label (Fig. 5).

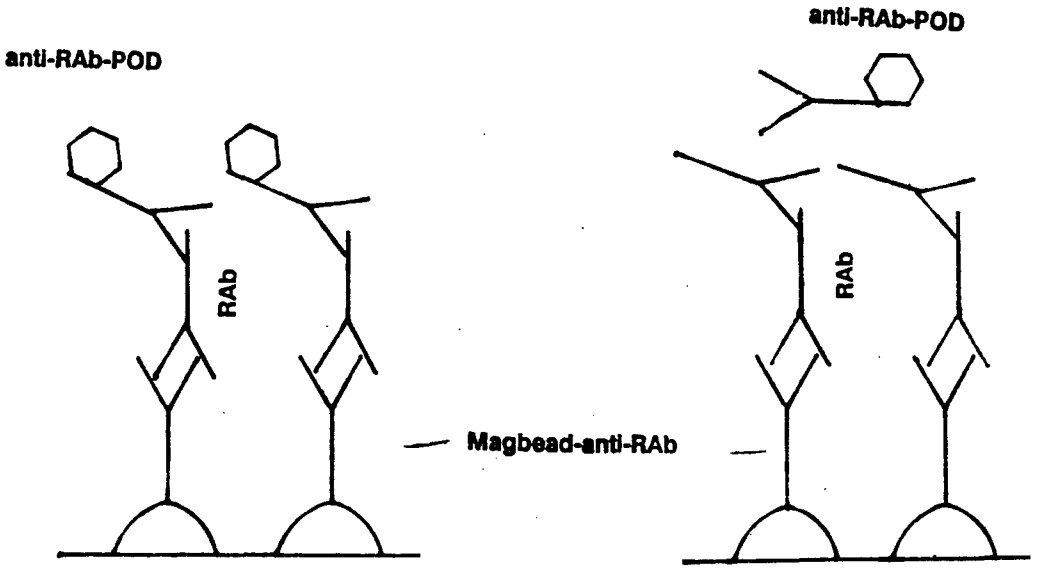

Figure 5. a. Schematic representation of total binding of anti-RAb-POD. b. Schematic representation showing blocking of RAb sites with unlabeled anti-RAb to determine non-specific binding of anti-RAb-POD.

3. RESULTS

3.1 Antibody Integrity.

Collected samples were quantitated by linear regression analysis of RAb standards made by 1:1 dilutions (100 ng/ml-3 ng/ml) RAb solution. ELISA data indicated that within this range, saturation of the RAb with anti-RAb does not occur (Fig. 6). On the average, 50% of the antibody remained intact.

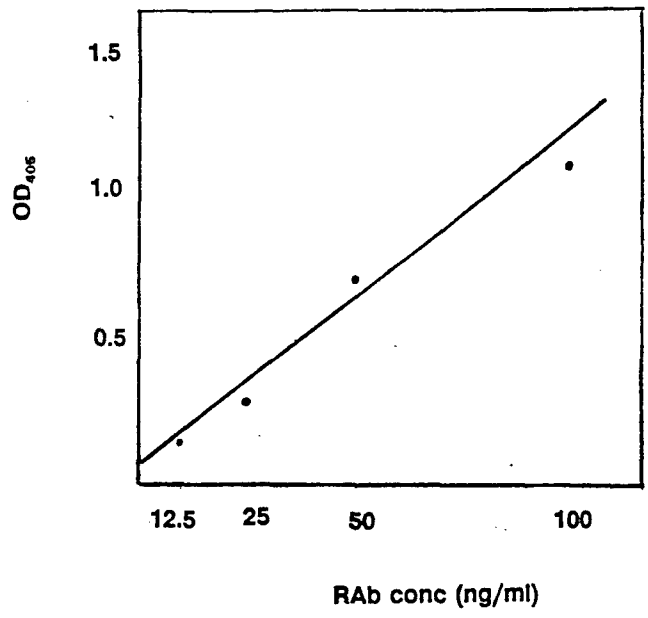

Figure 6. Representative RAb standard curve.

3.2 Antibody Binding.

Anti-BSA was aerosolized, collected and analyzed as previously described using BSA as a capture antigen. ELISA results indicated that anti-BSA retained its ability to bind to BSA after aerosolization (Table 1).

Table 1. Representative ELISA data for anti-BSA

Conditions	Column numbers/OD readings									
	1	2	3	4	5	6	7	8	9	10
No BSA capture	BLNK	0.03	0.02	0.03	0.03	0.03	0.02	0.03	0.02	0.01
BSA capture Standards (ng/well)	10	10	5	2.5	1.25	0.63	0.31	0.16	0.08	
	BLNK	0.79	0.76	0.68	0.46	0.31	0.21	0.15	0.12	0.10
AGI collected anti-BSA	BLNK	0.79	0.77	0.67	0.43	0.27	0.18	0.12	0.11	0.09

3.3 Antibody/Antigen Binding in Aerosol.

Aerosol particles were collected as described and quantitated as described. The clear package tape with collected magbead/RAb complex was affixed to the bottom of the plate. A control tape was likewise affixed to the bottom of a plate and assayed. Specific binding of RAb and anti-RAb was determined as illustrated in Figure 5. Table 2 shows the summary of OD readings from each of the experiments as well as the elimination of non-

specific interference caused by the tape/magbead interaction. The nonmagnetic tape OD indicated that insignificant droplets containing RAb bound to the tape during collection. The average of total OD_{405} signal was 0.320 while nonspecific signal from wells containing beads was 0.172, giving a specific signal of 0.148. This corresponded to 2.2 ng/ml of RAb collected and indicated that collision of particles had taken place.

Table 2. Summary of OD readings from three experiments.

Source of OD signal (less nonspecific binding due to tape)	
Total binding (magbeads+ captured RAb)=	0.320
Nonspecific binding (magbeads blocked with anti-RAb)=	0.172
Specific signal=	0.148

4. DISCUSSION

Antibodies bind with specificity to their respective antigens, whether it be of chemical or biological origin, and are very sensitive to blood proteins of other species. This binding specificity is the basis of detection in immunoassays such as ELISA. The attachment of the primary protein (RAb, BSA, magbead) in the polystyrene wells of the assay plate is non-specific and there exists the possibility for saturation of the well if too much protein is present, or for non-specific binding of secondary proteins (POD-anti-RAb). Since OD readings correlate to the amount of POD-anti-RAb label that binds in each well so it is of utmost importance to ensure that the label present is specifically bound to RAb. To eliminate the possibility of non-specific binding of label to the well, 1% skim milk (casein) was added to the label. This protein, as well as BSA, is routinely used for immunoassays. Casein was the blocker of choice since BSA was to be used as part of the experimental plan. Data collected for linear regression analysis showed decreasing amounts of RAb per well that correlated directly to decreasing OD_{405} readings, indicating that saturation of the well with RAb had not taken place and that POD-anti-RAb label binding was specific for the RAb, thus producing useful information for determination of RAb quantity by linear regression analysis.

In the first experiment, a percentage of RAb was shown to retain enough structural integrity to allow specific recognition by POD-anti-RAb label. However, since the label might bind to fragmented or denatured RAb, this alone does not conclusively show that an aerosolized Ab retains its antigen binding sites. Loss of antigen binding sites would render the Ab useless for testing or protection against a hazardous material.

For the second test, rabbit derived Ab was required so that the label would remain the same. Rabbit anti-BSA was chosen for the test solution since it is an inexpensive, non-hazardous means of testing and the capture antigen, BSA, readily binds to the walls of the well. ELISAs were performed using wells containing no BSA as controls. It would be expected that anti-BSA would bind to the walls of these wells; however, OD readings were only 5% of those containing BSA. It is surmised that the BSA in the wells attracted the anti-BSA with a greater affinity than the random binding of anti-BSA in the other wells. The greater OD readings indicated that the anti-BSA indeed bound specifically to BSA.

In the third test, four possible background interferences were determined. Since the bottoms of the wells were drilled out and clear tape used in its place, interference could be caused by the tape. The tape alone showed little signal at OD_{405} (0.0064) which is equivalent to > 0.05% of the average signal for assays; however, assays done in taped wells showed an OD up to 50% of the OD of assays done in the plastic well bottoms. This background was reduced by blocking compounds such as casein, but not eliminated. This phenomenon was consistent for all assays and was subtracted from the OD readings. RAb standards for linear regression analysis were also done on tape to insure similar assay conditions.

The second source of background was optical interference from the magbeads. ELISA assays done in standard microwells with magbeads indicated that about 10% of OD signal was due to optical interference of the magbeads.

The third possible interference resulted from the collector where non-specific attachment of RAb-containing droplets to the control (nonmagnetic) collector tape might occur. The collector was placed in a vertical position to eliminate settling of droplets on the tape. Comparison of OD readings from exposed (nonmagnetized) tape and clean tape from each experiment indicated no significant difference in the OD reading.

A fourth background interference was determined from the interaction of the magbeads with POD-anti-RAb label. Since magbeads have high affinity for Ab including the POD-anti-RAb label, a method was needed to show how much signal resulted from non-specific binding of label to the magbeads and how much was specific binding of RAb to the anti-RAb on the beads. Half of the sample was preincubated with unlabeled anti-RAb to block any exposed sites that could be occupied by POD-anti-RAb. The OD readings from blocked wells was subtracted from unblocked OD readings, to ensure that the OD reading was resulted only from RAb binding. It was determined that wells contained an average of 217 pg RAb. Collision of aerosolized particles did occur although under very controlled and exact conditions.

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